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# The Endogenous Exposome

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## Abstract

The concept of the *Exposome*, is a compilation of diseases and one's lifetime exposure to chemicals, whether the exposure comes from environmental, dietary, or occupational exposures; or endogenous chemicals that are formed from normal metabolism, inflammation, oxidative stress, lipid peroxidation, infections, and other natural metabolic processes such as alteration of the gut microbiome. In this review, we have focused on the *Endogenous Exposome*, the DNA damage that arises from the production of endogenous electrophilic molecules in our cells. It provides quantitative data on endogenous DNA damage and its relationship to mutagenesis, with emphasis on when exogenous chemical exposures that produce identical DNA adducts to those arising from normal metabolism cause significant increases in total identical DNA adducts. We have utilized stable isotope labeled chemical exposures of animals and cells, so that accurate relationships between endogenous and exogenous exposures can be determined. Advances in mass spectrometry have vastly increased both the sensitivity and accuracy of such studies. Furthermore, we have clear evidence of which sources of exposure drive low dose biology that results in mutations and disease. These data provide much needed information to impact quantitative risk assessments, in the hope of moving towards the use of science, rather than default assumptions.

### Keywords

Endogenous Exposome; DNA damage; stable isotopes; mutagenesis; risk assessment

In 2005, Chris Wild brought forward the concept of the "Exposome" [1]. He suggested that together with genomics, metabolomics, proteomics, and transcriptomics, we need to also understand relationships between life-time exposures to chemicals and disease. This concept was further explored several years later by Lijoy and Rappaport [2] and by Chris Wild [3], who pointed out that the assessment of exposures should not be restricted to chemicals entering the body from air, water, food, smoking, etc., but should also include internally generated toxicants produced by the gut flora, inflammation, oxidative stress, lipid peroxidation, infections, and other natural biological processes. In other words, we must focus upon the 'internal chemical environment' arising from all exposures to bioactive chemicals from within and outside the body.

This review will focus on recent advances in our understanding of endogenous DNA damage arising from the internal environment, and how it compares with external exposures. Advances in analytical methods have vastly changed our ability to accurately measure

biomarkers such as DNA adducts and protein adducts over the past two decades. When this is coupled with exposure to stable isotope labeled chemicals that cause identical DNA damage, we now can accurately compare the exposures that arise endogenously, with those coming from environmental, occupational and life style chemical exposures. This has important implications for understanding exposure responses, such as causality of mutations, cancer, disease and aging. Likewise, it begins to explain why mutations do not extrapolate to zero when studied at very low exposures. Rather, they often reach thresholds that appear to be driven by the *Endogenous Exposome*. The field of low dose mutagenesis, rather than high dose studies for hazard identification, has been grossly understudied, but is of great importance for the advancement of science-based risk assessment.

Research related to endogenous DNA damage and its relationship to a variety of chemical exposures has been a major focus of our laboratory for the past two decades. This review will cover research on abasic sites, oxidative DNA damage and several known human carcinogens that form exogenous adducts identical to endogenous DNA adducts, what the exposure-response relationships are for identical endogenous and exogenous DNA damage, and how this knowledge of the *Endogenous Exposome* helps us understand exposure-responses for mutations and disease, as well as informing science-based risk assessment.

## Apurinic/apryrimidinic sites

Apurinic/apryrimidinic (AP) sites are known to be one of the most prevalent types of endogenous DNA lesions (Table 1.). Endogenous AP sites in cellular DNA are partly derived from spontaneous depurination and depyrimidination of normal and unstable modified bases (e.g., N7-methylguanine (N7-meG) and N3-methyladenine (N3-meA)). In 1973, the rate of spontaneous AP site formation was first estimated to be 10,000 sites/cell/day using the depurination rate of DNA at 70°C and physical chemistry [4].

Twenty five years later, we directly demonstrated that AP sites are generated at 1.54 AP sites/$l0^6$ nucleotides/day (~9,000 AP sites/cell/day) at 37°C and pH 7.4 using aldehyde reactive probe [5]. In addition to spontaneous hydrolytic base loss, AP sites are also generated by the base excision repair pathway. An AP site serves as an intermediate DNA lesion during the repair of several modified bases, [6–8]. Our accumulated results indicate that the steady-state level of AP sites is approximately 30,000 lesions per genome in mammalian cells and tissues [9]. It is important to point out that these endogenous AP sites are likely oxidized deoxyribose [10]. Endogenous hydrogen peroxide, one of the major endogenous reactive oxygen species, generates hydroxyl radicals though the Fenton reaction with ferrous ($Fe^{2+}$) ions loosely attached to the N7 position of guanine and preferentially oxidizing the adjacent deoxyribose to generate various oxidized deoxyriboses, resulting in oxidized AP sites [11–13],. Since deoxyribose lesions are hard to quantitate with high sensitivity and specificity, a steady-state level of oxidized deoxyribose and their biological importance are largely unknown even though they are among the most abundant endogenous DNA lesion in living organisms. The regular AP sites, derived from spontaneous depurination and DNA glycosylase reactions, are cytotoxic through DNA replication blockage. In addition, they are mutagenic through translesion DNA synthesis. Previous

articles have extensively reported mutational potential and spectrums of AP sites in cells by transfection of exogenous DNA harboring AP sites.

We utilized an endogenous gene to better understand mutagenicity of AP sites in vertebrate cells under more physiological conditions. DT40 cells (chicken B cells) that naturally express $O^6$-alkylguanine DNA alkyltransferase (MGMT) were continuously exposed to very low doses of methylmethane sulfonate (MMS). These conditions allowed cells to repair mutagenic $O^6$-methy-2′-dexoyguanosine ($O^6$-mdG) before DNA replication and generate AP site-specific mutations [14]. We found a hockey-stick dose response curve with steady-state levels of mutations. Approximately half of the mutations induced by the low concentrations of MMS were transversion mutations at mainly adenine positions and the remaining half were deletions or insertions. These results suggest that N3-meA-derived AP sites likely cause transversion mutations and possibly deletion and insertion mutations through N-methylpurine-DNA glycosylase function, or via spontaneous depurination. While AP sites in genomic DNA sounds like unwelcome DNA lesions, some of the endogenous AP sites are essential for normal biological function such as cytosine methylation. Activation-induced deaminase (AID) initiates immunoglobulin diversity through deamination of cytosine to uracil [15, 16]. The uracil is excised by uracil-DNA glycosylase to generate AP sites, which cause mutations by translesion DNA synthesis and initiate recombination in immunoglobin genes [7]. In contrast to the beneficial property of AP sites, constitutive expression of AID (e.g., under chronic inflammation) causes an increase in global accumulation of uracil, 5-hydroxymethyluracil and possibly AP sites in non-immunogloblin genes, leading to mutations and cancers [7, 17, 18]. In summary, tightly controlled AP site formation and repair are beneficial for normal biological functions, however, imbalanced repair of AP sites leads to an increase in mutations above the threshold level.

## Reactive Oxygen Species

Endogenous DNA adducts have been identified in cellular DNA from cultured cells, tissues of animals and humans. As shown in Table 1, a majority of endogenous DNA damage appears to be derived from oxidative stress [9]. Apurinic/apyrimidinic (AP) sites are the most abundant, spontaneous DNA lesions. One of the characteristics of AP sites suggests that they may be oxidized deoxyribose and exist in cells under normal physiological conditions [9]. DNA single strand breaks (SSBs), which occur adjacent to oxidized AP sites through DNA repair mechanisms, or by spontaneous cleavage, should therefore be among the most profuse endogenous DNA lesions. However, due to technical difficulties in quantitating SSBs with high sensitivity and specificity, accurate steady-state levels are not well characterized to date. The next three most abundant endogenous DNA lesions are N7-(2-Hydroxyethyl)G, N7-(2-Oxoethyl)G, and 8-oxodG [9]. All three of these base adducts are caused by oxidative DNA damage due to either lipid peroxidation products or reactive oxygen species (ROS). Among various endogenous ROS, hydrogen peroxide ($H_2O_2$) is the most diffusible and plentiful, and are mainly produced by mitochondria under physiological conditions. In addition to mitochondria-derived $H_2O_2$, oxidative demethylation by histone demethylase (e.g., LSD1 and LSD2) produces $H_2O_2$ and formaldehyde, two major sources of endogenous DNA damage. These two reactive endogenous molecules are produced in

close proximity to genomic DNA, suggesting the $H_2O_2$ resulting from oxidative demethylation of histones could more efficiently damage DNA than mitochondria-derived $H_2O_2$. In fact, it has been reported that when LSD1-mediated demethylation occurred, oxidative DNA lesions were increased [19, 20]. Surprisingly, data have demonstrated that LSD1-mediated local oxidative DNA damage and its repair mechanisms work as a driving force in transcription initiation [19, 20]. This suggests that oxidative DNA damage could be beneficial for certain biological functions.

Previously, our lab demonstrated that the formation of single strand breaks, AP sites, and 8-oxo-dG followed a biphasic or polynomial dose response in $H_2O_2$-treated HeLa cells at concentrations ranging from 60 to 20,000 uM [21, 22]. To better understand the association between very low amounts of $H_2O_2$-induced oxidative base damage (*Biomarkers of Exposure*) and mutation events (*Biomarkers of Effect*), we conducted *thymidine kinase (tk) gene mutation* assays and 8-oxodG assays in human lymphoblastoid (TK6) cells exposed to $H_2O_2$ at concentrations ranging from 1 to 56.6 uM. As with MNU [23], $H_2O_2$ induced a hockey-stick dose response indicative of a threshold (Figure 1). We also found that $H_2O_2$ induces 8-oxodG with a hockey-stick dose response (unpublished results). The results indicate that $H_2O_2$ increases the frequency of mutations when oxidative DNA lesions are increased above spontaneous oxidative DNA damage. This implies that spontaneous mutations could be derived from oxidative DNA lesions.

In addition to direct oxidative DNA damage, ROS indirectly induces uracil and 5-hydroxymethyluracil from cytosine and 5-hydroxymethylcytosine (down-stream products of 5-methylcytosine) by enzymatic deamination though activation-induced deaminase (AID) function. AID-mediated deamination was observed under chronic oxidative stress, including chronic inflammation caused by bacterial/viral infection [17]. This suggests that uracil and 5-hydroxymethyluracil should be recognized as oxidative stress-associated DNA lesions.

## Vinyl Chloride

Vinyl chloride (VC) is a widely used chemical that was shown in the 1970's to induce hepatic angiosarcomas in workers. Human epidemiology and animal carcinogenicity studies lead to its classification as a human and rodent carcinogen [24–29]. While VC is used in industry to produce polyvinyl chloride (PVC) production, it also is present in tobacco smoke, and is found at Superfund sites due to microbial dechlorination of perchloroethylene and trichloroethylene [30–34]. VC requires metabolic activation by CYP450 2E1 to produce chloroethylene oxide (CEO), which covalently binds to DNA to induce four DNA adducts, while the secondary metabolite chloroacetaldehyde, alkylates primarily proteins [35–38].

The major DNA adduct [39] that is formed from the reaction between CEO and DNA is 7-(2-oxoethyl)guanine (7-OEG). This adduct lacks miscoding properties and it is removed from DNA primarily by chemical depurination. The exocyclic DNA adducts that are induced by VC, such as $N^2$,3-ethenoguanine (εG), 1-$N^6$-ethenodeoxyadenosine (εdA), and 3,$N^4$-ethenodeoxycytosine (εdC), have been studied in greater detail due to their promutagenic activity during DNA synthesis [40–42].

Previously, exocyclic endogenous VC DNA adducts [43–49] and more recently 7-OEG [50] have been detected in tissues of unexposed rats and/or humans, and have been shown to arise from lipid peroxidation and oxidative stress [51–54]. Several studies demonstrated the endogenous εdA and εdC formation in unexposed rodents by $^{32}$P-postlabeling [55]. While control liver DNA had 0.4–5.8 εdA/$10^9$dA and 0.6–40 εdC/$10^9$dC, lung and kidney had 1–2 εdA/$10^8$dA and 5–12 εdC/$10^8$dC; 2.5–3.5 εdA/$10^8$dA and 8–12 εdC/$10^8$dC, respectively. The $t_{1/2}$ of εdA was reported to be 24 h by Ham et al. [56], while 0.8 εdA/$10^8$dA was detected in control and A*ag* null mouse liver and 0.1 εdC/$10^8$dC in control and 0.7 εdC/$10^8$dC in A*ag* knockout mouse lung. In our laboratory, a new nano-UPLC-MS/MS method was developed to measure endogenous and exogenous εdA formation in rats exposed to1100 ppm [$^{13}C_2$]-VC for 5 days (6 hr/day) (Gao, unpublished data). While endogenous εdA was 4.9±0.6/$10^8$dA, [$^{13}C_2$]εdA was 5.1±0.6/$10^8$dA in [$^{13}C_2$]-VC exposed rat liver following exposure (Table 2).

*In vivo* formation of εG has been measured by GC-NICI-MS [57–60], immunoaffinity coupled-GC/HRMS [45, 48, 49, 61] and LC-MS/MS [62]. Fedtke et al. [59] reported the formation of εG that could not differentiate between endogenous and exogenous sources in rats exposed to 600 ppm VC for 5 days. While the concentration of εG was found to be the highest in liver, followed by the kidney and lung, the persistence was ~30 days. Being able to distinguish between endogenous and exogenous DNA adducts in the same animals were demonstrated for the first time by Morinello et al., using stable isotope labeled VC to determine the molecular dose of εG in rats exposed to 0, 10, 100 and 1100 ppm [$^{13}C_2$]-VC for one or four weeks [45, 48, 49, 61]. Rapid increases of [$^{13}C_2$]-εG adducts in hepatocyte liver DNA was reported between 10 and 100 ppm exposures, followed by a lower slope in adduct formation at 1100 ppm, thought to be the result of partial saturation of metabolic activation of VC. While endogenous εG was present in liver and brain, exogenous εG was detected in liver only. Similar results were reported by Mutlu et al. [62] for the formation of endogenous and exogenous εG in liver DNA (4.1±2.8 endogenous εG/$10^8$G and 18.9±4.9 [$^{13}C_2$]-εG/$10^8$G). In contrast, both endogenous 7-OEG and [$^{13}C_2$]-7-OEG were detected in several organs, including liver and brain in rats exposed to 1100 ppm [$^{13}C_2$]-VC [50] This suggests that not only liver, but the other organs are able to form low amounts of CEO, which results in lower [$^{13}C_2$]-7-OEG adduct formation, it is believed that VC is metabolized in each organ by CYP450, rather than arising via the circulation of higher liver-based CEO formation in the body.

When identical endogenous and exogenous DNA adducts are formed, the only way to derive accurate $t_{1/2}$ data is to conduct stable isotope exposures so that exogenously formed adducts have a higher mass than the corresponding endogenous adducts. In the earlier studies on VC DNA adducts without the use of stable isotope exposures, clearly determining how many DNA adducts came from normal cellular processes and how many were from the stable isotope exposures was not possible. This is well demonstrated in Table 2. The number of endogenous adducts do not change with time, as they are continuously formed and are thought to be at steady-state concentrations. To determine $t_{1/2}$ data, one must focus on the stable isotope labeled adducts that came from the exposures. For VC adducts, one can see that εdA has very rapid repair, with no labeled adducts being detectable after 2 weeks post

exposure, the shortest time post exposure in this study. [$^{13}C_2$]-7-OEG had a $t_{1/2}$ of 4 days, while [$^{13}C_2$]-$N^2$,3-εG had a $t_{1/2}$ of 150 days [40, 62]. This is not thought to be active DNA repair. Rather, it most likely represents loss due to cell death and cell replication.

## Ethylene/Ethylene Oxide

IARC reviewed the epidemiology of occupational exposure of humans and considered that ethylene oxide was *Carcinogenic for Humans* and that there was sufficient evidence of carcinogenicity in experimental animals [63] While not yet studied with stable isotope exposures, ethylene oxide (EO) is also formed endogenously. The endogenous production of ethylene results from oxidative stress [64] and from gut microflora [65]. Endogenous ethylene circulates in the blood and is metabolized to EO in the liver [66], which also circulates in the body. Endogenous EO forms N7-(2-hydroxyethyl)guanine adducts as shown in Table 1. Endogenous EO is also exhaled in human breath 24/7. Thus, we all have endogenous exposure to EO, a second known human carcinogen. *Hprt* mutations have also shown nonlinear responses following inhalation exposure of rats to ethylene oxide [67].

## Isoprene

Isoprene is the predominant hydrocarbon in the environment, being formed by both deciduous and conifer trees, as well as plants, with 10–100 ppb carbon being present in forest canopies. It also formed endogenously in humans and animals. Human production of isoprene has been calculated to be 0.15 μmol/kg/hr, resulting in blood concentrations between 15 and 70 nmol/L and breath concentrations in the range of 10–30 nmol/L, with 2–4 mg exhaled per day per individual [68]. Thus, all humans have relatively high endogenous exposure to isoprene daily. Isoprene is metabolized to two epoxides and a diepoxide that are genotoxic. These can form DNA adducts [69, 70]. Isoprene is carcinogenic in mice and rats, but has not been evaluated in human epidemiology studies [71]. To date, no studies of isoprene DNA adducts in animals exposed to stable isotope-labeled isoprene have been conducted, for comparisons for endogenous and exogenous formation.

## Endogenous and Exogenous DNA Adducts from Alkylating Agents

DNA alkylation can cause stalled replication, mispairing and DNA strand breaks ultimately leading to mutations or cell death [72, 73]. While these modes of DNA damage are known to be prominent key events in carcinogenesis, it has also been exploited as the anticancer therapy for a long time [73]. Alkylating agents exert their mutagenic and genotoxic effects by forming adducts with the N- and O-atoms in DNA bases [74]. The different proportions of adducts at oxygen versus nitrogen in DNA depend upon the nature of the reactive moiety [75]. Compounds such as methylmethane sulfonate (MMS) that have a high Swain Scott constant (s value) target highly nucleophilic centers within DNA (e.g., N7-G, N3-A) yielding N7-mG and N3-mA [76]. Low s value alkylating agents such as methylnitrosourea (MNU) alkylate O atoms (e.g., $O^6$-mdG) more efficiently than those with high s values. Though the alkylations at O atoms are formed at a much lower rate compared to N-alkylations, the resulting $O^6$-mdG adducts are highly promutagenic and can result in mismatches during DNA replication leading to mutations [77]. Alkylation at the $O^6$ position of guanine and $O^2$ and $O^4$ positions of thymine induces GC to AT, AT to GC and AT to TA

mutations, respectively [78]. Formation of DNA alkyl adducts may result from both endogenous as well as environmental alkylating agents. The properties of some alkylating agents (e.g. MMS and MNU) that react directly with DNA and trigger highly mutagenic effects makes them a popular model in laboratories for low-dose risk assessment and other mutagecity studies [79].

The major source of endogenous methylation is S-adenosylmethionine (SAM) which is a key compound in transmethylation, aminopropylation and transsulfuration metabolic pathways within cells [80]. It can act as a weak DNA methylating agent by forming adducts with DNA in a non-enzymatic manner [80]. The methylation pattern of SAM mimics MMS and acts by SN2 reaction, therefore, yielding more N7 and N3 adducts than the $O^6$ adducts. The ability of SAM to act as a methyl donor in a nonenzymatic reaction could contribute to the background mutation rate [80]. Endogenous DNA alkylations are considered to be a major contributor to the total background levels of DNA adducts (Table 1) [81]. Endogenous methyl adducts reflect part of the ever-present carcinogenic load in biological systems. The use of highly sensitive LC-MS/MS and isotope labeled compounds provides a tool to accurately identify and differentiate the origins of exogenous and endogenous DNA adducts. Using this sensitive approach, an earlier study from our group reported for the first time, minor methyl adducts at $N^2$-dG and $N^6$-dA positions. Both showed a linear dose response with $N^2$-methyl-dG having several-fold higher amounts than $N^6$-methyldA. However, $N^2$-methyl-dG and $N^6$-methyl-dA were found to be minor alkylation products compared to the N7-mG and $O^6$-mdG [82].

For alkyl-DNA adducts, $O^6$-mdG is considered to be the main contributor to point mutations. However, their low number and highly efficient repair mechanism makes their detection and quantification at low doses quite challenging [83, 84]. In a study by Brink et al., the $O^6$-mdG amounts in rat liver DNA were found below the limit of quantitation, but above the limit of detection (3.7 adducts per $10^9$ nucleotides) [83]. Georgiadis et al. were able to detect $O^6$-mdG in 70% of maternal and 50% of cord blood buffy coat samples at mean levels of 0.65 and 0.38 adducts/$10^8$ nucleotides, respectively, by using a new ELISA type assay [85]. The exquisite sensitivity of stable isotope nano-UPLC-MS/MS adduct detection offers opportunities for the development of biomarkers of exposure. Whereas biomarkers of exposure are expected to be linear down to zero unless removed by DNA repair, biomarkers of effect (e.g. mutations) can only be interpolated back to the spontaneous or background numbers of mutations. Recently, we conducted studies to compare the dose response relationships of MNU-induced DNA adducts with previously published mutation frequencies [23, 79] at low exposures. When identical endogenous DNA adducts are formed, the only way to determine the molecular dose of the exogenous DNA adducts is to utilize stable isotope-labeled chemicals and ultra-sensitive mass spectrometry. We utilized [$D_3$]-MNU, so that adducts formed by the exposure were three mass units higher than the endogenous DNA adducts. This permitted accurate quantitation of both N7-mG and $O^6$-mdG at attomol concentrations. The lowest two doses of [$D_3$]-MNU were measureable, but did not significantly increase the total number of endogenous and exogenous $O^6$-mdG. These molecular doses of $O^6$-mdG were consistent with the previous doses that resulted in a threshold for point mutation studies. This demonstrated that very low amounts of exogenous

$O^6$-mdG did not significantly increase the total $O^6$-mdG until ~10-fold higher doses of [$D_3$]-MNU were used for exposures.

When exogenous and endogenous N7-mG were measured in the same cells, the endogenous amounts of N7-mG were ~20-fold greater than that formed by the [$D_3$]-MNU at the lower end of exposure. Thus, much higher numbers of endogenous N7-mG adducts were present than were $O^6$-mdG adducts. The molecular dose of the major DNA adducts, N7-mG and $O^6$-medG, was quantified, over the same dose range as studied by Thomas et al. [23]. The exogenous DNA adducts were linear over the entire dose response and intersected with identical endogenous adducts (Figure 2) [86]. In contrast to DNA adducts, Thomas et al., found non-linear responses in point mutations, with a no-observed genotoxic effect level (NOGEL) of approximately 0.075uM and the lowest observed genotoxic effect level (LOGEL) of 0.1uM [23]. Combining these data, it can be concluded that only $O^6$-medG adducts of $\geq 1.8/10^8$ dG were effective in producing significant increases in mutations in AHH-1 cells. In contrast, at very low exposures, the biology that results in mutagenesis is driven by endogenous DNA damage, including identical endogenous DNA adducts.

It is also likely that DNA repair represents yet another mechanism for non-linear threshold response curves for alkylating agents [23, 87]. For example, EMS administered to mice in a single high dose caused mutations, but when the same total dose of EMS was given to the mice over 28 days, no increase in mutations was present [88]. Acrylamide induces increases in micronuclei at high doses, but not at low doses administered to mice [89]. Another recent example of this is the paper on radiation from the Engelwald laboratory at MIT, where a single high exposure to x-irradiation caused increased micronuclei, but the same total dose administered over 4 weeks did not induce any increase in micronuclei [90].

## Formaldehyde

Formaldehyde is classified as a known human and animal carcinogen according to IARC [91], following inhalation exposure. Formaldehyde exposure from occupational and environmental sources is very common. Formaldehyde can covalently bind to DNA, proteins and other cellular nucleophiles, forming formaldehyde derived DNA adducts [92–96], DNA-DNA crosslinks, DNA-protein crosslinks (DPCs) [97–103], and DNA-glutathione adducts [104] that, if not repaired or hydrolyzed, can lead to mutagenesis and carcinogenesis.

What is less understood is that formaldehyde is formed endogenously in all living cells [9]. The endogenous presence of formaldehyde and the high concentrations normally found in cells also result in the formation of identical DNA adducts and crosslinks to those formed by exogenous exposure. Because there is endogenous formaldehyde resulting in the same DNA damage, it is challenging to develop biomarkers of formaldehyde exposure and to understand its toxicity. Our laboratory has employed stable isotope-labeled formaldehyde exposures to afford the ability to differentiate between formaldehyde molecules of endogenous and inhaled exogenous origin. Exposure to stable isotope labeled [$^{13}CD_2$]-formaldehyde and the associated endogenous formaldehyde can readily be measured using nano-ultra performance liquid chromatography-tandem mass spectrometry (nano-UPLC-

MS-MS, limit of detection reaches 1.25 amol) [82, 105–108]. Because the hydroxymethyl DNA adducts of formaldehyde are chemically unstable, they must be reduced to a methyl adduct using cyanoborohydride prior to analysis. This approach is shown in Figure 3.

The primary genotoxic effects of formaldehyde are thought to result from the formation of DPCs. Increased DPCs were detected in nasal mucosa of rats exposed to formaldehyde by inhalation at concentrations greater than 6 ppm [109]. However, there were no detectable DPCs in the bone marrow of normal rats exposed to formaldehyde at concentrations as high as 15 ppm [110]. Similarly, DPCs were found in the nasal turbinates and anterior lateral wall/septum of non-human primates (NHPs) following exposure to as little as 0.7 ppm formaldehyde, while no DPCs were detected in the bone marrow at concentrations as high as 6 ppm [111]. Further studies indicated that protein adducts and DPCs were not detected in the bone marrow of rats exposed to formaldehyde at concentrations as high as 10 ppm [112], even when the degradation of formaldehyde was inhibited by glutathione depletion [98]. However, rapid decreases in formaldehyde induced DPCs were observed in cell culture models [113–115], but no accumulation of DPCs were observed in rats after multiple days of exposure [116]. Furthermore, evidence indicates that a major portion of formaldehyde induced DPCs were lost from lymphocytes through spontaneous hydrolysis, rather than being actively repaired [101].

The inability to distinguish between exogenous and endogenous formaldehyde associated DNA damage is a clear short coming for all previous studies on formaldehyde-induced DPC formation measured following formaldehyde exposures. This includes the potassium-SDS precipitation and chloroform/iso-amyl alcohol/phenol extraction [109, 117], both of which are not only unable to distinguish between endogenous and exogenous formaldehyde, but also cannot differentiate formaldehyde DPCs from DPCs induced by other cross-linking endogenous or exogenous chemicals.

The structures of formaldehyde-induced DNA adducts *in vitro* have been known for decades [93–96] and include the unstable DNA adducts $N^2$-hydroxymethyl-deoxyguanosine ($N^2$-HOMe-dG), $N^6$-hydroxymethyl-deoxyadenosine ($N^6$-HOMe-dA), and $N^4$-hydroxymethyl-deoxycytosine ($N^4$-HOMe-dC) [118–120]. $N^2$-HOMe-dG is the main DNA mono-adduct induced by inhaled formaldehyde, which together with DNA protein crosslinks and toxicity-induced cell proliferation are thought to play important roles in mutagenesis and carcinogenesis. However, similar numbers of endogenous $N^2$-HOMe-dG and $N^6$-HOMe-dA are present in most tissues [105].

In a study of formaldehyde DPC formed between deoxyribose nucleosides and amino acids or their oligos, we found that dG-lysine was the most formed cross-link, but that it very rapidly (2–5 minutes) underwent hydrolysis [121]. The second most formed cross-link was dG-cysteine. This cross-link was also shown to be the most stable. We have now evaluated the dG-cysteine, dG-glutathione, and a peptide from MGMT that also forms a dG-cysteine cross-link. When these were analyzed for hydrolytic degradation, we found that $N^2$-HOMe-dG was the dominant product (data unpublished). This demonstrates that $N^2$-HOMe-dG represents an excellent biomarker for both spontaneous DPC hydrolysis and direct adduction of inhaled formaldehyde to DNA.

In studies using inhalation exposure to [$^{13}CD_3$]-formaldehyde, we clearly demonstrated that exposures of labeled [$^{13}CD_2$]-formaldehyde induced [$^{13}CD_2$]-$N^2$-HOMe-dG adducts in nasal epithelium of rats and monkeys [105–107]; however, these adducts were not detected in tissues distant to the site of contact, including lung, liver, spleen, mononuclear white blood cells, or bone marrow [105]. In contrast, endogenous $N^2$-HOMe-dG and $N^6$-HOMe-dA adducts were readily detected in all tissues examined [105]. When [$^{13}CD_4$]-methanol was administered to rats, [$^{13}CD_4$]-$N^2$-HOMe-dG and [$^{13}CD_4$]-$N^6$-HOMe-dA adducts were formed in kidney and bone marrow [108], suggesting that the $N^6$-HOMe-dA adducts are only formed when methanol is metabolized to formaldehyde within a cell.

Our most recent study that exposed rats to 2 ppm [$^{13}CD_3$]-formaldehyde for up to 28 consecutive days further demonstrated [$^{13}CD_2$]-$N^2$-HOMe-dG accumulation, with 28 days being the approximate time to reach steady-state concentration, and the $t_{1/2}$ for the repair/loss of [$^{13}CD_2$]-$N^2$-HOMe-dG *in vivo* being ~202 hrs. As with our previous studies [105–107], endogenous formaldehyde-induced $N^2$-HOMe-dG adducts were observed in all tissues analyzed; however, exogenous formaldehyde-induced [$^{13}CD_2$]-$N^2$-HOMe-dG adducts were only detected in the nasal respiratory epithelium DNA of rats exposed to [$^{13}CD_2$]-formaldehyde by inhalation, providing compelling evidence that inhaled formaldehyde does not reach tissues distant to the sites of initial contact in an active form. A nonhuman primate study utilizing up to 6 ppm [$^{13}CD_3$]-formaldehyde exposures for two 6hr/day exposures had the ability to find one [$^{13}CD_3$]-$N^2$-HOMe-dG adduct in 10 billion unmodified dG, but no exogenous adducts were found in bone marrow [107]. A similar sensitivity was used to evaluate mononuclear white blood cells in the 28-day rat study, but none were found. This raises important questions regarding how inhaled formaldehyde could cause leukemia [122].

By conducting studies at durations ranging from a single 6 hr exposure, to 5 days (6hr/day), and up to 28 consecutive days (6hr/day), it was clear that exogenous $N^2$-HOMe-dG adducts had not reached steady-state concentrations, while endogenous $N^2$-HOMe-dG adducts were at steady-state due to continuous intracellular formation through normal metabolic pathways. When the National Research Council reviewed the 2010 EPA IRIS Cancer Risk Assessment, they pointed out that understanding what exposures to formaldehyde increased the intracellular amounts of total formaldehyde above the endogenous represented critical data for risk assessment [123].

In summary, the use of stable isotope formaldehyde exposures has provided new insight and tools for science-based risk assessment. The data generated in these studies provide pivotal information for understand the toxicity and carcinogenicity of formaldehyde, as well as the biological plausibility of leukemia induction following inhalation exposure to environmental formaldehyde. The NRC Review of the Environmental Protection Agency's Draft IRIS Assessment of Formaldehyde in 2011, strongly suggested that such data be incorporated into a revised risk assessment [123].

## Acetaldehyde

Acetaldehyde is a highly reactive 2-carbon aldehyde that is a ubiquitous environmental pollutant with a variety of potential human exposures ranging from occupational activities,

consumer products, lifestyle choices (food/alcohol/cigarette consumption), and environmental sources [124]. Acetaldehyde is listed as reasonably anticipated to be a human carcinogen by the National Toxicology Program and as a human carcinogen from the metabolism of ethanol by the International Agency for Research on Cancer [124, 125]. Acetaldehyde is also a metabolite of vinyl acetate, a common industrial chemical used in the manufacture of a variety of consumer and industrial applications. Acetaldehyde is endogenously produced as a by-product of cellular respiration and metabolism [126–128]. Complicating the risks associated with acetaldehyde exposure is that the primary route of detoxification of acetaldehyde to acetate is via a well characterized polymorphic enzyme, aldehyde dehydrogenase 2 (ALDH2) metabolism of acetaldehyde to acetate [129].

Acetaldehyde is a direct, DNA reactive mutagen causing a number of genotoxic effects including DNA adducts, DPC, DNA-protein crosslinks, DNA-DNA crosslinks, single strand and double strand breaks, micronucleus formation, mutations, and sister chromatid exchange (reviewed in [130]). The two primary DNA adducts formed and measured as biomarkers of exposure are $N^2$-ethylidene-dG and 1,$N^2$-propano-dG. The $N^2$-ethylidene-dG adduct is formed following the direct attack of acetaldehyde at the $N^2$ position of 2′-deoxyguanosine. In contrast, the 1,$N^2$-propano-dG adducts can arise from either the reaction of crotonaldehyde, or by two molecules of acetaldehyde catalyzed by the amines of histones, or amino acids found in the nucleus [131, 132]. Similar to the $N^2$-OHMe-dG adduct of formaldehyde, $N^2$-ethylidene-dG is unstable ($t_{1/2}$ = 20 min) at the nucleoside level and requires reduction with a strong reducing agent to the more stable $N^2$-ethyl-dG for analysis [133]. Site directed mutagenesis studies have shown that the stable $N^2$-ethyl-dG adducts are weakly mutagenic, while 1,$N^2$-propano-dG adducts have a higher mutagenic potential [134, 135]. The 1,$N^2$-propano-dG adduct can exist in either the ring opened or closed positions, from which the ring opened form may react to form either DNA-DNA or DNA-protein crosslinks (reviewed by [136]).

The $N^2$-ethylidene-dG and 1,$N^2$-propano-dG adducts are the most commonly measured biomarkers of exposure to acetaldehyde. Recent studies [132, 133] in cell culture using stable isotope exposures of [$^{13}C_2$]-acetaldehyde have revealed that both endogenous and exogenous lesions can be formed. Our recent data [133] reporting $N^2$-ethylidene-dG adduct formation in TK6 cells exposed to a [$^{13}C_2$]-acetaldehyde concentration range ≥4.5 orders of magnitude illustrates several key points. Using the stable isotope approach, the endogenous adducts remained relatively constant across the dose range (~2–3 adducts/$10^7$ dG), with similar results to what has been observed in our [$^{13}CD_2$]-formaldehyde [105–107] and [$D_3$]-MNU studies. At low exogenous exposure conditions (≤10 μM), the amount of endogenous $N^2$-ethylidine-dG adducts dominates over much smaller numbers of exogenous adducts (Figure 4). At high exposure conditions (≥250 μM), exogenous adducts dominated over endogenous adducts. The stable isotope exposures allowed for ~50x difference in the lowest concentration (1 μM), with observable formation of exogenous DNA adducts over the sum (endogenous + exogenous) of the adducts at 50 μM, as would have been observed using unlabeled acetaldehyde. Importantly, the exogenous adducts had a linear dose-response across the dose range, while the sum of the adducts showed a non-linear response that demonstrates a threshold in DNA adduct formation at 50 μM and below. This nonlinearity

was also observed for micronucleus formation rates and cell survival, with statistically significant increases over background occurring at 1000 μM [133]. A more in-depth study investigating the chromosomal and gene level effects of acetaldehyde using 4 and 24 hour exposures showed a non-linear dose-response for micronucleus formation and the induction of mutations at the thymidine kinase (TK $^{-/-}$) loci between 50 and 100 μM in TK6 cells [137].

These studies utilizing stable isotopes and large exposure ranges support the hypothesis that that endogenously produced reactive species, including acetaldehyde, formaldehyde and lipid peroxidation are always present, and constitute a majority of the observed background DNA damage following low exposures to these compounds.

## Discussion

As shown in this review, recent advances in analytical chemistry have greatly increased the accuracy, sensitivity and identification of endogenous DNA damage. In addition, new sources and forms of endogenous DNA damage are being identified that arise from oxidative stress and inflammation. As these new sources and types of damage are discovered, new associations with mutations and diseases are becoming known. Thus, the role of the *Endogenous Exposome* is rapidly gaining broader acceptance. When such DNA damage is coupled with the use of stable isotope exposures, highly informative data can be collected that place experimental, life style, environmental, occupational and medical exposures in perspective with identical endogenous DNA damage. Thus, Chris Wild's *Exposome* concept has been shown to be complex, but doable. As such, the effects of both endogenous and exogenous exposures can be placed in better perspective [1, 3]. It is likely that evaluating the *Exogenous Exposome* over a life-time will be even more difficult than similar studies on the *Endogenous Exposome.*

A recent study using the *Rag2*$^{-/-}$ mouse model together with *Helicobacter hepaticus* demonstrated that in the absence of the innate immune system, hypochlorous acid formed by myeloperoxidase from neutrophils formed large amounts of 5-chlorocytosine that was strongly associated with inflammatory bowel disease [138]. Etheno and other exocyclic DNA adducts also have been examined in animal and human studies by Bartsch and colleagues. These studies range from age-dependent increases in etheno DNA adducts in liver and brain of OXYS rats compared to age-matched Wistar rats [139] Likewise, iron and copper accumulation in the liver has been associated with increased numbers of exocyclic DNA adducts, as has inflammation and alcoholism [60, 140–142]. As additional research on inflammation and cancer is done, it is likely that additional endogenous forms of DNA damage will be shown to play roles in disease. Furthermore, improved chemical identification and quantitation of newly discovered forms of endogenous DNA damage are highly likely. Thus, the *Endogenous Exposome* is expected to grow as sources of new DNA damaging electrophiles are identified and their consequences known.

Genetically altered cell lines, such as the DT40 cells that have single gene knock outs, permit identification of critical pathways for DNA repair [156]. Such studies have already resulted in the development of animal models that demonstrate the importance of the

*Endogenous Exposome*. For example, it has become more evident that endogenous aldehydes play a large role in human diseases. While the importance of ALDH2 in the clearance of acetaldehyde has been well understood for many years, Ridpath et al., demonstrated that FANCD2 was an essential gene for DT40 cells to survive exposures to formaldehyde and to acetaldehyde [143]. The interplay of these two gene families has become clearer with the availability of *Aldh2*$^{-/-}$ and *Fancd2*$^{-/-}$ knockout mice that develop leukemia both with and without ethanol exposure due to the inability to metabolize acetaldehyde and repair the associated DNA lesions [144]. Further studies using aged *Aldh2*$^{-/-}$ *Fancd2*$^{-/-}$ mice that did not develop leukemia showed that the mice are predisposed to developing aplastic anemia due to the accumulation of DNA damage within the hematopoietic stem and progenitor cell pool [145]. This relationship has been further explored in humans by increased bone marrow failure in Fanconi Anemia patients who have the defective *ALDH2* gene variant [146]. Similar animal models are being developed and utilized to examine comparable susceptibilities to endogenous and exogenous formaldehyde. A current review on Fanconi Anemia has examined possible mechanisms for the susceptibility to endogenous aldehydes [147]. What has not been recognized is that bone marrow of nonhuman primates has ~ 5 times higher endogenous formaldehyde $N^2$-HOMe-dG adducts than other tissues [94]. At this time, no data are available to compare this with human bone marrow. If present in humans, it could represent an endogenous pathway that increases the susceptibility of bone marrow toxicity in Fanconi Anemia patients.

What is clear, is that endogenous DNA damage is associated with disease. However, controlling diseases arising from the *Endogenous Exposome* will certainly be a challenge. Nevertheless, using data such as the research presented in this review has major implications for mutagenesis, cancer and science-based risk assessment. It has long been known that mutations do not go to zero at very low exposures, yet the default science policy for genotoxic chemicals is currently to linearly extrapolate risks down to 1 additional cancer in a million individuals. Such risks have never been demonstrated in humans or animals. It is well recognized that mutations represent major key events in carcinogenesis. As such, mutations represent critical information for carcinogenic risks.

The research conducted by Moeller, et al. on acetaldehyde provides the clearest evidence that endogenous DNA adducts can have major effects on the total formation of both endogenous and exogenous DNA adducts. It also demonstrates the effect of endogenous DNA adducts on the induction of mutations and demonstrates that low exogenous exposures do not increase mutations [133]. Figure 4 clearly shows the contribution of endogenous (black) and exogenous N2-ethylidene-dG adducts (red). Our biostatisticians were asked when does the total adduct load of both endogenous and exogenous adducts become significantly greater than endogenous adducts alone. As discussed earlier, it was only at 50 μM [$^{13}C_2$]-acetaldehyde and higher that the exposures increased the total DNA adducts. Of even greater importance, micronuclei and cell death were not increased over background until the cells were exposed to 1000 μM [$^{13}C_2$]-acetaldehyde [133]. Likewise, the studies of *Aldh2*$^{-/-}$ and *Fancd2*$^{-/-}$ knockout mice clearly demonstrate that endogenous aldehyde exposure induces bone marrow toxicity and leukemia, with no exogenous exposure.

As more and more studies show thresholds for mutagenesis and a greater understanding of why such thresholds are present, defaults should be replaced with scientific data when it is available. This review has certainly demonstrated that many forms of endogenous DNA damage are present in the cells of our bodies. The data in Table 1 represent our results over the past two decades, but additional forms of endogenous DNA damage are known [140, 141, 148, 149]. Penman and Crespi published one of the seminal papers on background mutations in untreated human cells [150]. They evaluated TK mutations in 87 independent studies and *hprt* mutations in 34 independent studies and found that mutations ranged from 1–4 mutations per $10^6$ cells. These background mutations can arise from the *Endogenous Exposome*, as well as errors of DNA polymerases.

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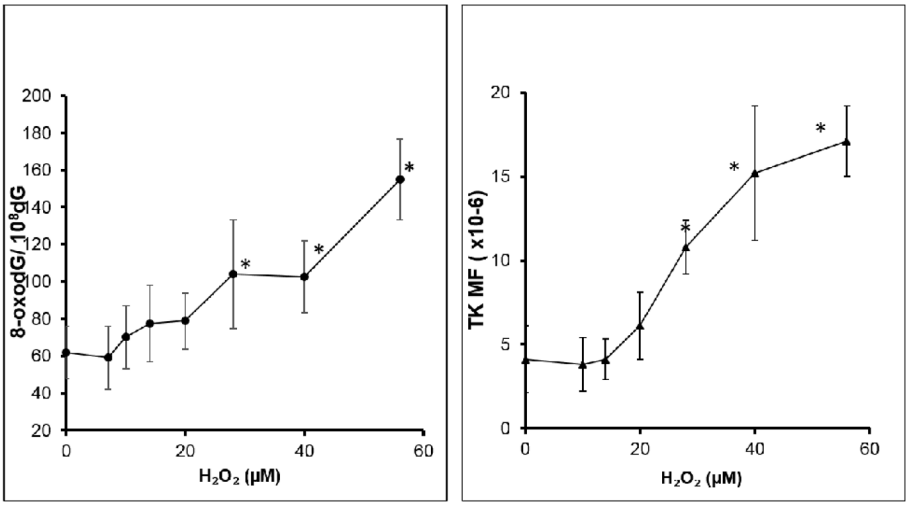


**Figure 1.**

Dose-response relationships of $H_2O_2$ (30 min exposure) in TK6 cells with respect to (A) 8-oxodG adducts (B) Mutation frequency (MF) in TK forward mutation assay (MF). Data represent Mean +/− SD * *p<0.05;* one-way analysis of variance (ANOVA) with Dunnet's test were employed to test for doses statistically significant from control.

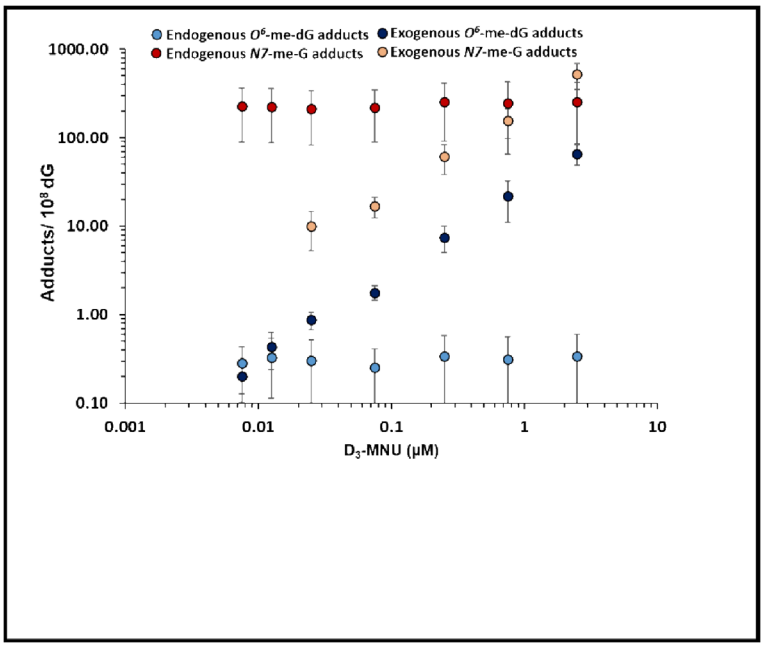


**Figure 2.**

Endogenous versus exogenous adducts in AHH-1 cells exposed to [$D_3$]-Methylnitrosourea (0.0075uM to 2.5uM) for 1h. The endogenous and exogenous $O^6$-me-dG and *N7*-me-G adducts at each exposure concentration are plotted on a log versus log scale. Exogenous adducts from samples with no detectable amounts are not shown. Data represent Mean +/− SD. Statistical comparison between the sum of adducts and the endogenous mean was conducted using a *t*-test (*$p<0.05$) to determine doses when the amount of total adducts become significantly higher than the identical average endogenous adducts. This dose was ≥ 0.75uM for *N7*-me-G and ≥0.025uM for $O^6$-me-dG.

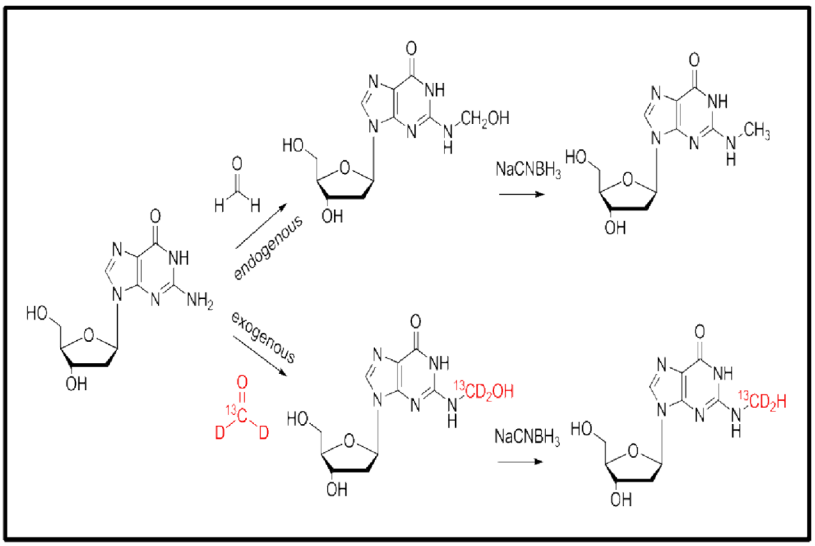


**Figure 3.**
Approach for stable isotope versus endogenous $N^2$-HOMedG

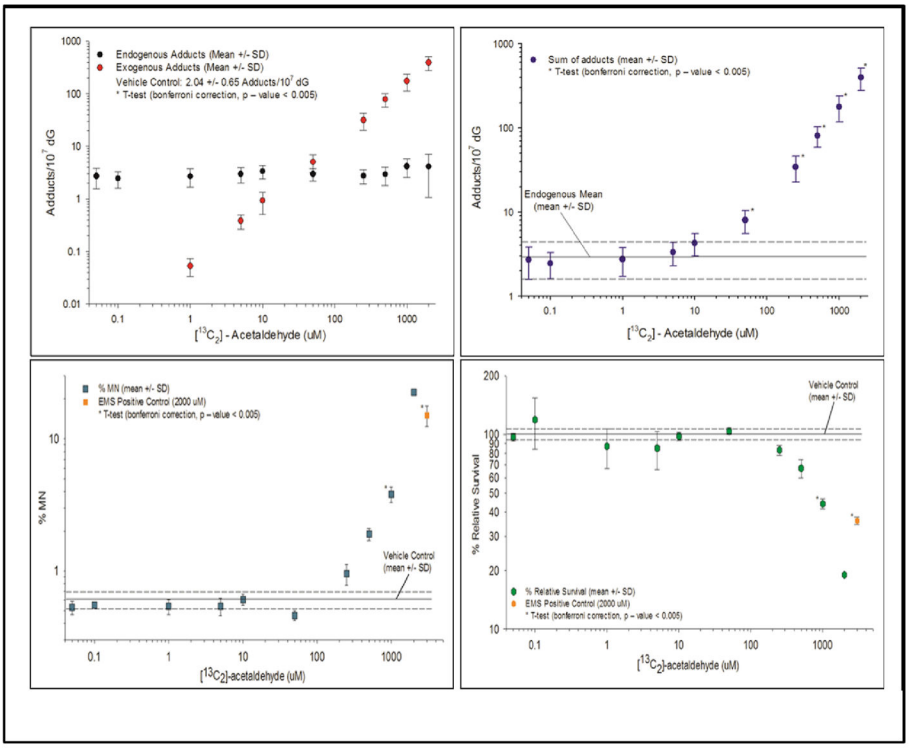


**Figure 4.**

When do exogenous DNA adducts result in a total endogenous plus exogenous adducts that is significantly greater and mutations are significantly greater than controls. Adapted from Moeller, et al., *Toxicol. Sci.* **133:** 1–12, 2013

## Table 1

### The Endogenous Exposome

Steady-State amounts of Endogenous DNA Damage

| Endogenous DNA Lesions | Number per Cell |
|---|---|
| Abasic sites | 30,000 |
| OHEtG | 3,000 |
| 7-(2-Oxoethyl)G | 3,000 |
| 8-oxodG | 2,400 |
| Formaldehyde | 1,000–4,000 |
| Acetaldehyde | 1,000–5,000 |
| 7-Methylguanine | 2,300 |
| AcrdG | 120 |
| $M_1$dG | 60 |
| $N^2$,3-Ethenoguanine | 36 |
| 1$N^2$-Etheno dG | 30 |
| 1$N^6$-Ethno dA | 12 |
| $O^6$-Methyl dG | 2 |
| **Total** | **40,000+** |

**Table 2**

Endogenous and Exogenous DNA adducts in Liver DNA from rats exposed to 1100 ppm [$^{13}C_2$]-VC

| | Endogenous | Exogenous | Endogenous | Exogenous | Endogenous | Exogenous |
|---|---|---|---|---|---|---|
| | 7-OEG/$10^5$ G | [13C2]-7-OEG/$10^5$ G | $N^2$,3-εG/$10^8$ G | [$^{13}C_2$]-$N^2$,3-εG/$10^8$ G | 1,$N^6$-εdA/$10^8$ dA | [13C2]-1,N6-εdA/$10^8$ dA |
| 2h post-exposure | 0.2±0.1 | 10.4±2.3 | 4.1±2.8 | 18.9±4.9 | 4.9±0.6 | 5.1±0.6 |
| 2wks post-exposure | 0.1±0.03 | 0.4±0.3 | 3.7±3.1 | 14.2±4.2 | 8.6±0.9 | ND |
| 4wks post-exposure | 0.2±0.04 | 0.1±0.06 | 3.1±1.0 | 16.9±1.6 | 6.2±1.3 | ND |
| 8wks post-exposure | 0.2±0.07 | ND | 3.7±1.5 | 13.2±2.5 | 4.1±0.5 | ND |

ND, not detected